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Detection of EMMPRIN in the Trigeminal Ganglion in Rats

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INTRODUCTION

The overall neuronal function is based on precise neuronal connections, which are considered to be mediated by common recognition strategies with guidance cues provided by adhesion molecules in combination with diffusible repellents and attractants[1]. It has been reported that extracellular matrix proteins, members of the immunoglobulin (Ig) superfamily, integrins, and cadherins can modulate the growth, differentiation and survival of various types of nerve cells[2-9].

Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) is a highly glycosylated membrane protein belonging to the Ig superfamily[10,11]. EMMPRIN is composed of two immunoglobulin domains in the extracellular region, a single transmembrane domain and a short cytoplasmic domain containing 39 amino acids. The strong expression of EMMPRIN in the mouse and the rat has been detected in the brain as well as other organs, including testis, kidney, heart, liver, and small intestine[10,12-15], suggesting that EMMPRIN in mammals has diverse biological functions.

The trigeminal nerve conveys information through three main divisions, the ophthalmic, the maxillary, and the mandibular. A majority of the trigeminal sensory neurons have their cell bodies clustered in the trigeminal ganglia (TG)[16]. In mammals, the proportion of unmyelinated fibers to myelinated is much lower in trigeminal nerve branches than in spinal nerves. This unique characteristics in the trigeminal nerve

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may influence its response to injury. Therefore, an in-depth understanding of trigeminal molecular mechanisms may be required so that the diverse and complex clinical problems can be solved.

Preliminary experiments demonstrated that EMMPRIN is active in expression at postnatal periods. The present study was investigated for the first time the localization of EMMPRIN and its function in the TG. Furthermore, changes in EMMPRIN expression was examined in the TG after surgical cut of branches of the trigeminal nerve as a model of nerve injury.

MATERIALS AND METHODS

Animals and Tissue Collection

Neonatal rats: Thirty Sprague-Dawley rats at postnatal days 4, 7 and 10 were sacrificed and TGs were taken. A few of them were fixed in 4% paraformaldehyde for immunohistochemical analysis. The remaining TGs were immediately frozen for reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analysis.

Adult rats: Adult Sprague-Dawley rats weighing 180-220g were anesthetized by intraperitoneal injection of ketamine (50 mg/ml). All of the left mandibular and maxillary teeth were extracted using a forcep and the lingual and mylohyoid nerves were cut. Animals were sacrificed at 3 and 5 days after the cut by perfusing them with 4% paraformaldehyde, followed by TG isolation. The right TG were used as a control. The isolated tissues were post-fixed, embedded in paraffin, and cut into sections for immunohistochemical staining. The remaining TGs were immediately rapidly frozen.

RT-PCR

Total RNA was extracted from the TGs using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription reactions were performed as follows. After the RNAs were treated in DNase I, they were mixed with oligo(dT)12-18 and heated at 70 ℃ for 10min. For the generation of 1st strand cDNA, the RNAs mixture was then added to solution composed of dNTP each, dithiothreitol and 1st strand buffer, followed by incubation at 42℃ for 60 min and subsequent incubation for 15 min at 70℃. The PCR primers used in the present study and their GeneBank accession numbers were listed in Table 1.

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Table 1. The sequence of PCR primers

	Forward (5′→ 3′) Reverse (5′→ 3′)	Annealing Temp (℃)	Product Size (bp)	Ref *
GAPDH	Forward 5′ CCA TGG AGA AGG CTG GGG 3′ Reverse 5′ CAA AGT TGT CAT GGA TGA CC 3′	65	195	AF106860.2
EMMPRIN	Forward 5′ CGG AAT TCC GGA ACA CGC CAG TGA GG 3′ Reverse 5′ GCG GAT CCA CAG GAG TGG AGG CAG ACG 3′	57	553	NM_012783.1

* Genebank accession number

Western blot analysis

The TGs were resuspended in a lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM thenylmethylsulfonyfluroide, 1% aprotinin, 1% leupeptin, 1 mM NaF], and then lysed using a homogenizer. The lysates were centrifuged to remove cell debris, and the supernatant was collected and frozen at -70℃ until further use. Protein lysates were boiled in a denaturing sample buffer and loaded onto 10% continuous gradient SDS-polyacrylamide gel for the transfer to nitrocellulose membrane (Amersham Pharmacia Biotech.). The membrane was blocked with TBS-T buffer [10 mM Tris-buffered isotonic saline (pH 7.0), 0.1% merthiolate, 0.1% Tween-20] containing 5% nonfat dry milk with shaking and incubated with 1: 200 goat anti-EMMPRIN primary antibody (Santa Cruze Biotech.) in TBS-T buffer containing 5% nonfat dry milk with mild shaking. The membrane was washed twice with TBS-T and reacted with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Bound antibodies were visualized using ECL (Amersham Pharmacia Biotech.).

Immunohistochemistry

For the immunohistochemical staining, the streptavidin-biotin-peroxidase method was used. Endogenous peroxidase in tissue sections was inactivated using 0.3% hydrogen peroxide. Sections were then reacted with primary antibodies; 1: 200 goat anti-EMMPRIN (Santa Cruze Biotech.) diluted in primary antibody diluent (Invitrogen), and incubated overnight at 4℃. They were then washed in phosphate-buffered saline (pH 7.4) for 10 min and incubated with biotinylated anti-goat immunoglobulin (Dako

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KIT LSAB2), followed by incubation with streptavidin-labelled peroxidase solution (Dako KIT LSAB2). 3-amino-9-ethy-carbazole (AEC) was used for visualization. Normal goat serum instead of primary antibody was used for negative control. All controls yielded negative results.

RESULTS

Expression of EMMPRIN mRNA in the TG during developmental stages

EMMPRIN mRNA was screened for its expression at three developmental time points (P7, P10 and adult). The transcripts were apparently reduced in adults (Fig. 1).

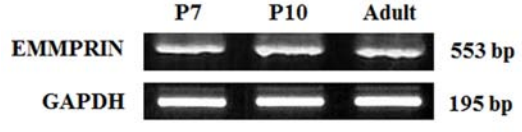

Fig. 1. An agarose gel image of RT-PCR products for EMMPRIN at three time points: postnatal days 7, 10 and adult. EMMPRIN mRNA appeared decreased in adults. This figure is a representative in triplicates.

Western blot analyses were carried out with a antibody against EMMPRIN in TG at four postnatal developmental time points (P4, P7, P10 and adult). Interestingly, the expression of EMMPRIN during the developmental process increased at P10, but decreased in adult (Fig. 2). Therefore, these data may reflect that high expression of EMMPRIN at early developmental stage in the TG may be related to axonogenesis.

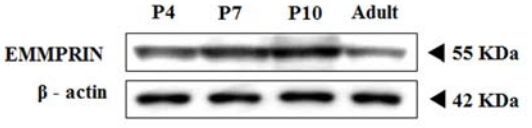

Fig. 2. Total protein from trigeminal ganglia in early postnatal periods and adults was analyzed by SDS-PAGE and immunoblotted with goat anti- EMMPRIN. The expression of EMMPRIN increased during TG development, followed by decreasing at adults. This figure is a representative in triplicates.

Alterations in expression of EMMPRIN in the TG by the trigeminal nerve injury.

To assess whether expression of EMMPRIN was altered as a result of the nerve damage, the left side of the teeth were extracted at 3 and 5 days prior to the isolation of the ganglia. The time points were determined to detect the modulation of mRNA levels during the initiation of transcriptional changes associated with the regenerative response. As a result, increased levels of mRNA were found at day 5, compared with the control side (Fig. 3).

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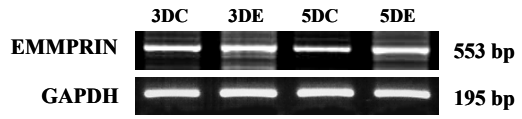

Fig. 3. An agarose gel image of RT-PCR products for EMMPRIN at 3 and 5 days after the trigeminal ganglion injury. Increased levels of the mRNAs is seen 5 days after the nerve cut. This figure is a representative in triplicates. C: control, E: experiment

Immunohistochemistry of EMMPRIN in the TG Development.

To localize EMMPRIN in nerve cells of the TG, sections of the TG were immunohistochemically stained using antibodies against EMMPRIN. Its localization was determined at three postnatal time points: postnatal days 7 and 10 and adult. The reactivity was observed in all the developmental time points. The immunoreactivities were demonstrated at bodies of both small and large nerve cells (Fig. 4).

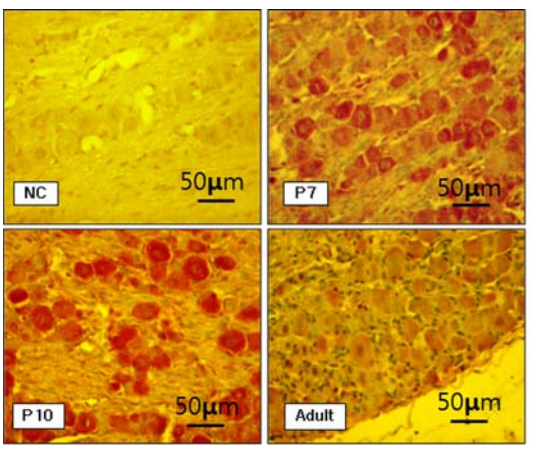

Fig. 4. Localization of EMMPRIN in the TG during the developmental time points (P7, P10 and adult). Strong immunoreactivity was demonstrated in nerve cell bodies at early periods, compared with that at adults. NC: negative control

Alterations in immunoreactivity of EMMPRIN in the TG after trigeminal nerve injury.

To determine whether EMMPRIN protein expression changes in the TG neurons after nerve injuries, branches of trigeminal nerves were cut. As shown in Fig. 5, the expression of EMMPRIN in the ganglia from the cut side was increased at 3 and 5 days, compared with the control side.

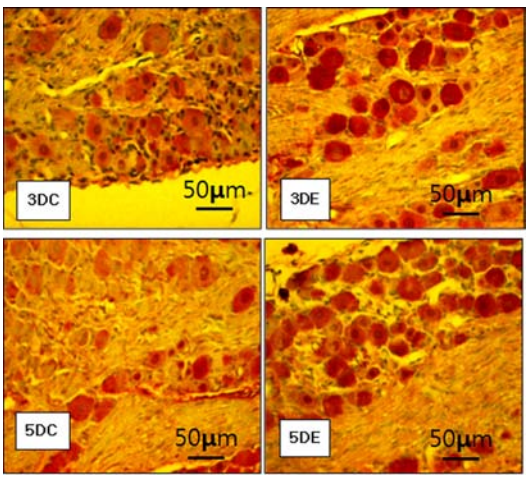

Fig. 5. Expression of EMMPRIN in the TG after the nerve injuries. Compared with the control, the cut side TG displayed the increased immunoreactivities at both 3 and 5 days. C: control, E: experiment

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DISCUSSION

The present study investigated the presence of EMMPRIN and its function in the TG using RT-PCR, Western blotting and immunohistochemical analyses. Expression of EMMPRIN in the organogenesis period of TG proposed that the protein has putative neural functions. EMMPRIN is a highly glycosylated transmembrane glycoproteins with two immunoglobulin domains in the extracellular region, a single transmembrane domain and a short cytoplasmic domain and form a subgroup in the immunoglobulin superfamily[11,17-19]. Many of studies support the role of EMMPRIN in tissue development and cell differentiation through MMP-inducing activity[20,21]. Glycosylation of EMMPRIN was shown to determine MMP-stimulating activity during development involving major tissue remodeling[17].

Although many studies investigated the function and the signalling pathway of EMMPRIN in several organs, its roles, especially in the nervous systems, have not been reported. In the present study, the analysis of mRNA and protein expression of EMMPRIN in the TG at the developmental time points (postnatal days 4,7 and 10) demonstrated that the transcripts were increased during the early developmental points. These increased expression of EMMPRIN during early growth periods may imply that axonal growth continues postnatally. Previous studies revealed that cadherin and its receptor of CAM is essential in the neuron differentiation and morphogenetic growth[22-24]. In fact, the axonal sprouting into the molar teeth in rats occurs postnatally. Meanwhile, the expression of EMMPRIN protein in the TG was decreased at adult period, comparing with the early postnatal periods. This difference may suggest that the peripheral pocesses of the TG were mature and stable, not needing active axon sprouting in adult.

In the nerve injury model, increased levels of EMMPRIN were found from RT-PCR and immunohistochemistry at 3 and 5 days after the nerve cut, compared with the control side. In previous studies, MMPs have essential role for tissue remodeling and axon regeneration in the nerve injury model. The increased EMMPRIN may increase the release of MMP protein for tissue modeling sequentially. Thus, it is possible that EMMPRIN in the nervous tissue may be involved in nerve regeneration, including axogenesis.

All together, EMMPRIN may be involved in cell differentiation at initial stage of development in the TG. Moreover, these results may imply a crucial role in developing and repairing physiology of neurons. Therefore, further studies

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are needed to elucidate its signalling pathway in the nervous system.

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국문초록

삼차신경절에서 EMMPRIN 탐색

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Extracellular matrix metalloproteinase inducer (EMMPRIN)은 두 개의 면역글로블린 도메인을 포함하는 고도로 당질화 된 막통과당단백이며 EMMPRIN은 CD 147, basigin 또는 neurothelin이라고도 불린다. 이전 연구들에서 EMMPRIN은 기관발생 동안 상피조직, 뇌 심실, 척수 및 dorsal root ganglion에서 발현되는 것으로 보고되어 왔다. 본 연구는 삼차신경절에서 현재까지 보고되지 않은 EMMPRIN의 존재 및 기능을 RT-PCR, Western blotting 분석 및 면역조직화학법을 통하여 구명하고자 시도되었다.

본 연구에서는 흰쥐 생후 4, 7, 10일 그리고 성체 시기의 삼차신경절을 추출하였으며, 신경 재생 모델로써 삼차신경으로부터 분지되어 나온 가지들을 외과적으로 절단한 후, EMMPRIN 발현을 조사하였다. mRNA와 단백질 수준에서 EMMPRIN 발현은 생후 4,7,10일 동안 점차 증가하였으며 성체에서는 감소되는 양상을 나타냈다. 면역조직화학법을 시행한 결과, EMMPRIN은 신경절세포의 세포질에 존재하였으며, 단백질 발현 변화를 확인할 수 있었다. 신경절단 3일과 5일째 삼차신경절에서 EMMPRIN 발현은 대조군에 비하여 실험군에서 mRNA와 단백질 발현이 증가하는 양상을 나타내었으며, 이는 면역조직화학법에서도 확인되었다.

이상의 결과는 EMMPRIN 발현은 삼차신경절의 신경재생과 발달에 관여함을 시사하였다.

주제어 : EMMPRIN, 삼차신경절, 발생

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